Thesis for doctoral degree (Ph.D.) 2018

B cell subsets in autoimmune disease

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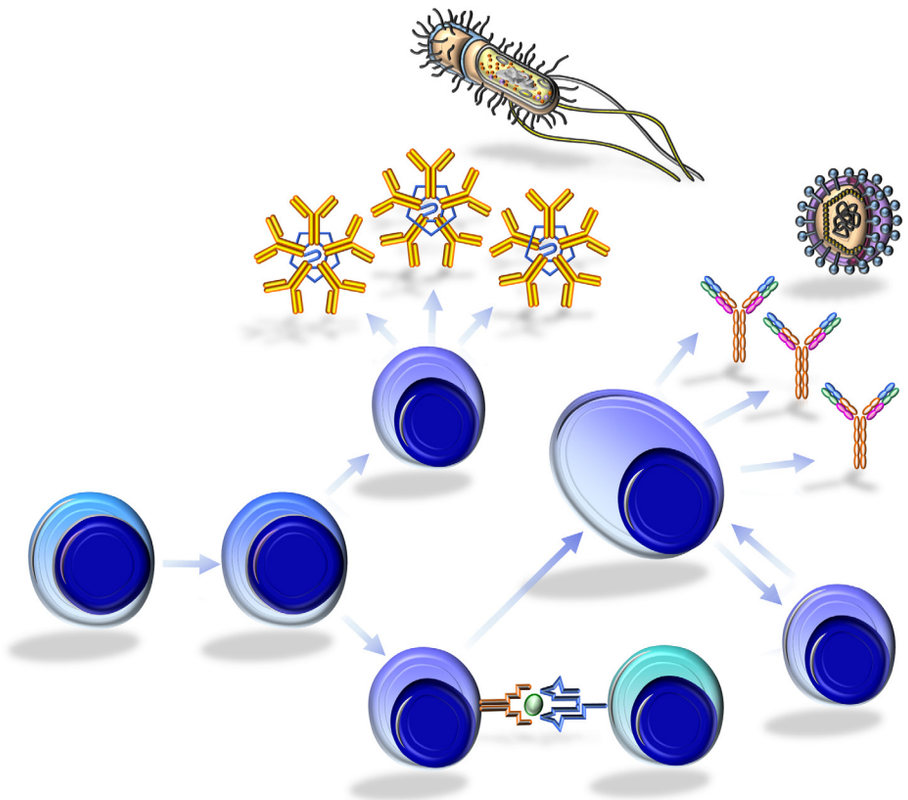

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B CELL SUBSETS IN AUTOIMMUNE DISEASE

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B CELL SUBSETS IN AUTOIMMUNE DISEASE

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Friday February 2nd 2018 at 09.00

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“We still do not know one thousandth of one percent of what nature has revealed to us.”

Albert Einstein

THIS THESIS IS DEDICATED TO MY BELOVED ONES

ABSTRACT

B lymphocytes are a type of white blood cells, belonging to the adaptive arm of the immune system and involved in creating immunological memory. B cells function in the humoral immune system by secreting antibodies which can bind pathogens to prevent them from doing further damage and to help other immune cells to target them. It is highly important that the immune system can distinguish self from none-self, since an immune response to self-antigens would cause the immune system to attack the hosts own healthy cells and tissues. The development of different B cells subsets is highly complex and both differentiation and proliferation is under strict transcriptional control. Defects in lymphopoiesis can lead to serious disorders like immunodeficiency, allergy, malignancy and autoimmunity.

In **Study I** we wanted to create a B1 cell-deficient mouse model by deleting the transcription factor *Arid3a* in a B cell-dependent manner. B1 cells are known to secrete natural antibodies which have shown to be atheroprotective and play other important roles in pathogenic conditions and diseases affecting a large number of people in the world. Our results demonstrate that *Arid3a* is required for specific immune responses and for the generation of normal cell numbers in a subset-dependent manner.

In **Study II** we were interested in understanding the function of germinal center formation in rheumatoid arthritis. We wanted to explain the involvement of antibody production and to target germinal center B cells, highly involved in the development and progression of this disease. Our results show that germinal center B cells are essential for experimental arthritis and targeting them could help when establishing more refined B cell-depleting therapeutics for clinical arthritis.

In **Study III** we wanted to understand the role of the adaptive immune system throughout the disease course of multiple sclerosis. B lymphocytes are known to be highly involved in influencing this disease, however the role of germinal center B cells has been unknown. To illuminate the function of germinal center responses, we induced experimental autoimmune encephalomyelitis in a murine model lacking germinal centers. We show that the functions of germinal center B cells is antigen-dependent and can both protect and promote disease.

In **Study IV** we deleted *Apoe* in a novel atherosclerotic murine model, in order to induce acute hypercholesterolemia in adult mice. Currently, there exists various atherosclerotic mouse models, however atherosclerosis differs between human and mouse and therefore we were interested in interrogating this disease in an inducible murine model. Our results demonstrate that the acute loss of *Apoe* triggers an autoimmune response, accelerating atherosclerosis.

LIST OF SCIENTIFIC PAPERS

I. Katrin Habir, Shahin Aeinehband, Fredrik Wermeling and Stephen Malin.
A role of the transcription factor Arid3a in mouse B2 cell expansion and peritoneal B1a generation
Front Immunol. 2017 Oct 24;8:1387. doi: 10.3389/fimmu.2017.01387

II. Albert Dahdah, Katrin Habir, Kutty Selva Nandakumar, Amit Saxena, Xu Bingze, Rikard Holmdahl and Stephen Malin.
Germinal center B cells are essential for collagen-induced arthritis
Arthritis Rheumatol. 2017 Oct 17. doi: 10.1002/art.40354

III. Albert Dahdah, Katrin Habir, Amit Saxena, Tomas Olsson, Rikard Holmdahl and Stephen Malin.
Antigen-dependent functions for Germinal Centers in Experimental autoimmune encephalomyelitis
Manuscript

IV. Monica Centa, Kajsa E. Prokopec, Manasa Garimella, Katrin Habir, Lisa Hofste, Julian Stark, Albert Dahdah, Chris Tibbit, Konstantinos A. Polyzos, Anton Gisterå, Daniel K. Johansson, Nobuyo N. Maeda, Göran K. Hansson, Daniel F.J. Ketelhuth, Jonathan Coquet, Christoph J. Binder, Mikael Karlsson and Stephen Malin.
Acute loss of Apolipoprotein E triggers an autoimmune response that accelerates atherosclerosis
Submitted

CONTENTS

LIST OF ABBREVIATIONS

Arid	A+T rich interactive domain
ACP	Anti-citrullinated protein
ACPAs	Anti-citrullinated protein antibodies
ANA	Anti-nuclear antibody
APC	Antigen-presenting cell
ApoE	Apolipoprotein E
B-ALL	B cell acute lymphoblastic leukemia
BCR	B cell receptor
Bregs	Regulatory B cells
Bright	B cell regulator of immunoglobulin heavy chain transcription
BTK	Bruton's tyrosine kinase
CIA	Collagen-induced arthritis
CII	Type II collagen
CLL	Chronic lymphoid leukemia
CLP	Common lymphoid progenitor
CNS	Central nervous system
CSR	Class switch recombination
CVD	Cardiovascular disease
DMARDs	Disease-modifying anti-rheumatic drugs
EAE	Experimental autoimmune encephalomyelitis
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorting
GC	Germinal center
GWAS	Genome-wide association studies
HDL	High-density lipoprotein
HSA	High heat stable antigen
HSC	Hematopoietic stem cell
IFN-γ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
ImmGen	Immunological Genome Project
LDL	Low-density lipoprotein
MHC	Major histocompatibility complex
MPP	Multipotent progenitor
MS	Multiple sclerosis
MZ	Marginal zone
NP-KLH	NP-Keyhole Limpet Hemocyanin
PAMPs	Pathogen-associated molecular patterns
PC	Phosphorylcholine
PCR	Polymerase chain reaction
PRRs	Pattern recognition receptors
RA	Rheumtoid arthritis
RT-PCR	Reverse transcription polymerase chain reaction
SHM	Somatic hyper mutation
SLC	Surrogate light chain
SLE	Systemic lupus erythematosis
SNPs	Single nucleotide polymorphisms
TdT	Terminal deoxynucleotidyl transferase
TGF-β	Transforming growth factor beta
TIR	Toll/IL-1 receptor
TLR	Toll-like receptor
TNF	Tumor necrosis factor
VLDL	Very low-density lipoprotein

AIMS OF THE THESIS

The aims of this thesis was to investigate the implication of B lymphocytes in different autoimmune conditions and to clarify the role of different mature B cell subsets in various inflammatory disorders using genetically modified murine models. Our specific scientific goals were to:

Study I Create and investigate a pure B1 cell-deficient mouse model by conditionally deleting the transcription factor *Arid3a* in a B cell-dependent manner.

Study II Clarify the function of germinal center B cells and antibodies in rheumatoid arthritis by studying collagen-induced arthritis in two germinal center-deficient mouse models.

Study III Understand the critical functions of B cells in the disease course of multiple sclerosis by inducing Experimental autoimmune encephalomyelitis in a murine model lacking germinal centers.

Study IV Investigate the development and early inflammatory response of autoimmune conditions and cardiovascular disease, by deleting *Apoe* in the adult mouse in order to induce acute hypercholesterolemia.

1 INTRODUCTION

1.1 THE IMMUNE SYSTEM

The immune system has a critical function in helping to protect us from pathogens. It consists of physical and chemical barriers, molecules, cells and tissues, coordinating immune responses against bacteria, viruses and other microorganisms [156]. When pathogens break through these barriers, an inflammatory response is almost immediately induced, characterized by heat, redness, local swelling and pain, due to dilation of the blood vessels and accumulation of proteins and cells at the site of the infection. The innate immune system is mainly activated at the site of infection where the pathogens invade the host, while the adaptive immune system is activated in peripheral lymphoid organs [1]. Together, both parts of the immune system work fast and efficiently to eliminate the foreign microbes [7].

1.1.1 The innate immune system

The first part of the defense against microbes and tissue injury is the innate immune system. When an infection occurs, the components of the innate immune system are rapidly activated upon encountering the pathogens to maintain a healthy microenvironment [156]. This first line of the immune system is generally not specific to certain pathogens, and depends therefore on molecules and cells recognizing conserved characteristics of pathogens that are not present in the host. The innate immune system is estimated to be able to recognize up to 10^3 different molecular patterns [6, 113] and reacts rapidly to any foreign pathogen encroaching the host. The molecules and cells of the immune system are many and become quickly activated when recognizing a foreign pathogen.

The innate immune cells and molecules include phagocytic cells, antigen presenting cells, natural killer cells, natural antibodies, the complement system, and physical barriers such as epithelial cells. Epithelial barricades, such as the skin and the interior mucus-covered surfaces in the lung and gut, provide protection against potential pathogens and mechanical and chemical damage [1]. These integral components of the innate immune system also produce chemokines and cytokines and have the ability to recognize and deal with danger signals. From time to time, microorganisms break though the epithelial surfaces, challenging the innate and adaptive immune system. For the immune system it is important to recognize and destroy the pathogens without damaging the host, and so the immune cells need to distinguish non-self from self [7, 156].

The soluble proteins of the complement system, circulating in the extracellular fluid and in the blood, are mostly inactive until an infection triggers an activation. The early components of the complement system are activated first and consist of the classical, alternative and lectin pathway, all of them activating the main component of the complement C3 [156]. Since these early components are proenzymes, the three pathways all lead to cascades of molecule cleavages, resulting in binding to the surface of foreign pathogens, thus helping to the next reactions in the immune system. The complement system was first discovered to be an effector arm of the antibody response, however complement is also activated without the presence of antibodies [39].

Humans and most of the mammalian species have membrane-bound glycoproteins called Toll-like receptors (TLRs), which are mainly expressed in myeloid cells, but also found in other cell types. TLRs recognize certain ligands, inducing inflammatory cascades [118]. Upon activation, cytoplasmic Toll/IL-1 receptor (TIR) homology domains signal and engage further molecules to activation. These receptors are together called pattern recognition receptors (PRRs), since they recognize certain structural patterns. The structures of the microorganisms recognized by the innate immune system are named pathogen-associated molecular patterns (PAMPs), but are not necessarily the final antigen to be presented by the antigen presenting cells (APCs) [116]. PRRs are able to induce phagocytosis, inflammation and maturation of APCs, the latter providing and important link between the innate and adaptive immune system [118, 160, 263].

B1 cells belong to a subset of B cells that produce antibodies that react against microbes passing through the intestine walls, amongst others, and are thus predominantly found in the mucosal tissues and the peritoneal cavity. These cells are considered innate-like lymphocytes [126], although B cells in general are a part of the adaptive immune system. In healthy mice, most of the IgM circulating in the blood, is secreted by B1 cells. These natural antibodies recognize carbohydrates present on many bacteria [1]. Additionally, phagocytic cells, like long-lived macrophages or short-lived neutrophils, are quickly at the site of inflammation when an infection occurs. They have receptors, such as Toll-like receptor (TLRs) at the cell surface, recognizing foreign pathogens and engulfing them.

After the phagocytic cells have taken up the pathogens, the newly formed phagosomes get acidified and lysosomes help to degrade the pathogens [156]. Macrophages secrete cytokines, like Tumor necrosis factor (TNF), and chemokines, like CXCL1, CXCL2, and CXCL5, attracting neutrophils, monocytes and dendritic cells to the location [254].

Once the dendritic cells pick up epitopes from the pathogens, they transport them to nearby lymph nodes, presenting them to lymphocytes and thus activating the adaptive immune system. In humans and other vertebrates, these immune responses are required to initiate the more specific adaptive immune responses [1].

1.1.2 The adaptive immune system

The second line of the immune system is the adaptive immunity, which is acquired throughout life, and activated after an immune response has been initiated. Compared to the innate immune system, the adaptive immune system is slow to react on the first exposure to a new foreign pathogen, however it is very specific and can respond to millions of different foreign antigens [7].

Lymphocytes, called B and T cells, are responsible for the specificity of the adaptive immune system. They circulate in blood and lymph, and are found in organs like spleen, thymus and lymph nodes. Lymphocytes are committed to a certain antigen or hapten, and after the first exposure to this antigen, they develop a memory that results in a quick and more effective response next time the lymphocytes encounter with the same antigen [156]. In humans, the adaptive immunity consists of about 2×10^{12} lymphocytes [7], usually only responding after the innate immune system is activated. The innate immunity depends on the previously mentioned PRRs, recognizing foreign immuno-stimulants and activating cascades of immune responses. Some of these cascades result in the production of extracellular signaling molecules promoting and activating the adaptive immune system.

Dendritic cells, present in almost all vertebrates, are professional APCs belonging to the innate immune system, recognizing and engulfing foreign pathogens where they infect the host, and then migrate to nearest peripheral lymph nodes to present epitopes directly to T lymphocytes. This initiates an adaptive immune response, resulting in T cells responding to the epitopes. Some of the activated T cells migrate to the site of the infection, helping other cells to defeat the pathogens, while other T cells stay in the lymphoid organs to help and activate B cells [156]. These B cells can terminally differentiate into plasma cells and secrete antibodies that spread throughout the body, coating the invading pathogens and helping the phagocytic cells to target them more efficient.

Both B and T cells develop from pluripotent hematopoietic stem cells (HSCs) located in the liver of the fetus and in the bone marrow of the adult, referred to as central lymphoid organs. T cell precursors then migrate to the thymus and develop further in that central lymphoid organ, while B cells develop from the hematopoietic stem cells (HSCs) in the hematopoietic tissues in liver and bone marrow [1].

Almost all lymphocytes fail to fully develop into a functioning B or T cell, whereas a small number migrate from the central to the peripheral lymphoid organs where they have the possibility to react to foreign invading agents, be activated, proliferate and mature into effector cells [7]. T cells can roughly be divided into cytotoxic and helper T cells, the first one terminating infected cells, and the latter one helping to active B cells, macrophages and cytotoxic T cells with the help of secreted local mediators, named cytokines, and costimulatory proteins on their surface. The binding of antibodies to the foreign epitopes also help other cells like macrophages to target them for phagocytosis.

As a part of the adaptive immune system, B cells play central roles in humoral immunity and protect, with the help of an enormous diversity of antibody specificities, against an almost unlimited variety of pathogens [7]. When B lymphocytes develop in the central lymphoid organs, they are committed to respond to a certain epitope, expressing it through cell-surface receptors that specifically fit to that antigen. When this particular antigen later is encountered by the B cell in a peripheral lymphoid organ, it binds to the receptor, leading to activation, proliferation and differentiation of the lymphocyte, making the adaptive immunity antigen-specific [7].

After the initial exposure with a pathogen, the adaptive arm of immunity creates a memory, being the reason why lifelong immunity to common diseases is developed. Comparing the primary immune response, the first exposure to a new foreign antigen, with the secondary immune response where the immune system has already created a memory, the primary immune response takes several days to react, rises exponentially and quickly, and then slowly declines. However, next time the immune system encounters with the same pathogen, the secondary immune response responds fast and strong, due to the immunological memory that has been created [7]. In order to prevent lymphocytes from responding to self-antigens, the lymphocytes are controlled during development. Lymphocytes hat show tendencies to bind to self are either induced to destroy themselves, get inactivated or suppressed, or their receptors are altered. Nevertheless, defects in lymphopoiesis, differentiation or function exist, resulting in severe disorders like immunodeficiency, malignancy, allergy and autoimmunity.

1.1.3 Autoimmunity

Autoimmunity is a condition where the adaptive immune system breaks down the tolerance to self-antigens, causing harm to organs or components of the blood, that is, it fails to differ between non-self and self, and thus attacks self-components instead of foreign pathogens. There are several hypotheses to why autoimmunity develops, however the main factors are environmental stimuli, infections, injuries and inheritance of susceptibility [1].

Rare mutations are also suggested to have a large impact on the development of autoimmune diseases [122, 276]. Studies in animal models and genome-wide association studies (GWAS) have further increased our knowledge about common polymorphisms of genes contributing to various autoimmune diseases [19, 44, 103, 162, 175]. Hundreds of gene polymorphisms associated to autoimmunity have been identified by GWAS [57, 178, 267], the majority of these genetic changes predicted to affect immune functions. The major histocompatibility complex (MHC) is a very gene-dense region, and MHC loci are known to be the strongest reported associations in most diseases [178].

Autoimmune diseases like systemic lupus erythematosus (SLE), rheumatoid arthritis (RA) and multiple sclerosis (MS) are characterized by the development of autoantibodies and the presence of autoreactive immune cells [275], and although our knowledge grows, as well as the increasing disease prevalence [95, 112], the etiology of several common autoimmune diseases remains unknown, due to heterogeneous and multifactorial reasons. Twins, especially monozygotic twins, have shown to be more prone to develop the same disease compared to other family members, elucidating the importance of inheritance and genetics in susceptibility to autoimmune diseases [17, 45, 60, 82, 174]. Other studies report epigenetics to play an important role in development of many autoimmune diseases [96, 134, 172, 242, 243].

Important studies propose B lymphocytes to be highly involved in autoimmune disease, especially those associated to humoral autoimmunity. Alterations in B cell signaling might initiate or promote autoimmunity, due to defects in genes involved in B cell function encoding signaling effectors, receptors and downstream transcriptional regulators of the BCR, cytokine receptors, CD40 or TLRs [111]. B lymphocytes express both innate pattern-recognition receptors like TLRs, and clonally rearranged antigen receptors, called B cell receptors (BCRs) [192]. Anti- viral antibody response are dependent on TLR signals from B cells through the adaptor protein MyD88 [105], however dual activation of TLRs and BCRs can also increase the risk of autoimmunity, since TLRs on B lymphocytes respond to endogenous antigens [192]. Both TLR and BCR activation have protective functions against infections, but also have potential to promote autoimmune disorders, thus these signaling pathways need to be strictly regulated.

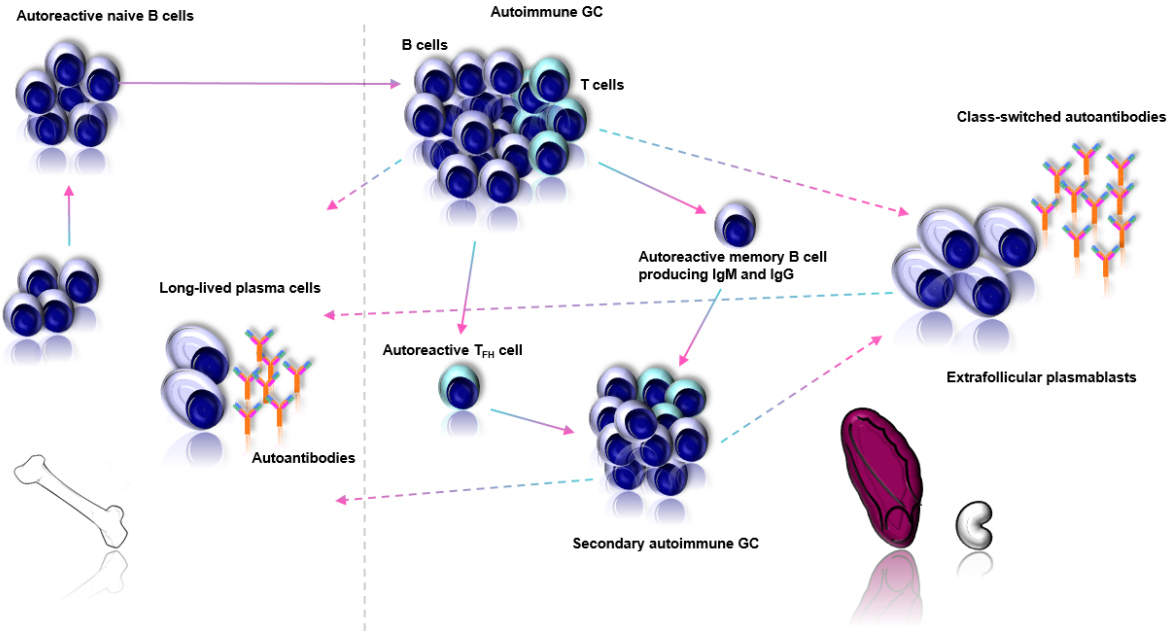

Figure 1. Schematic illustration of systemic autoimmunity.

Altered B cell signaling increases the risk of developing autoimmune disorders by modifications of negative and positive selection during B lymphopoiesis. Nevertheless, this does not immediately lead to autoimmune disease, since autoreactivity alone is rarely sufficient for developing disease. It is thought that formation of germinal center (GC) B cells that produce autoantibodies, generated from naïve or mature B lymphocytes, is a crucial second step for disease development. Subsequently, autoimmune GC B cells generate long-lived plasma cells, secreting class-switched autoantibodies, as well as memory B cells that are able to contribute in forming new autoimmune GCs and extra-follicular self-reactive B cells. It has also been suggested that autoimmune GCs promote the generation of autoreactive T follicular helper cells, which contribute to the formation of new autoimmune GCs [51, 189]. They could also have additional functions than solely providing help to B cells.

1.2 B CELL SUBSETS AND DEVELOPMENT

1.2.1 Early B cell development

During early embryonic development, pluripotent hematopoietic stem cells (HSC) migrate into the fetal liver. These HSCs can differentiate into B cells, as well as other immune cell types, populating the lung, epithelia and lymphoid tissues in the gut [237]. Fetal liver predominantly generate long-lived B1a cells and their precursors disappear in the early stages of life [28]. Short-lived B1b cells, derived from the HSCs in the bone marrow, have similar characteristics as the B1a cells. However, precursors of B cells generated in the bone marrow can not develop into B1a cells later in life. The majority of B cells in the adult are of the B2 type and are generated in the bone marrow, migrating to spleen and lymph nodes [87], organizing B cell follicles and developing into marginal zone B cells found in the marginal zone of the spleen. The bone marrow maintains the ability to repopulate the host throughout life, although the numbers of HSCs decrease with increasing age. The differences between hematopoietic development in fetal liver and adult bone marrow have been demonstrated by several important experiments [169]. After birth, the bone marrow (BM) becomes the major site for continuous B lymphopoiesis. Self-renewing HSC give rise to progenitor cells which undergo lineage commitment and develop through multipotent progenitors (MPP) followed by common lymphoid progenitors (CLP), pro-B, pre-B and immature B cells [5].

Gene rearrangements supports life-long generation of B cell repertoires [135], capable of recognizing antigens. The development of these repertoires critically depends on signaling molecules playing important roles in proliferation, differentiation, gene rearrangements, survival and apoptosis. Immunoglobulin gene rearrangement is important for B cell development and is regulated by several important transcription factors, such as PU.1, E2A, EBF and Pax5 [38, 54, 194, 199, 238].

Recent studies have shown that lineage skewing begins earlier than the CLP stage [107, 119, 138, 238, 270]. However B cells fully commit to the B lineage at the pro-B cell stage. This continuous B cell lymphopoiesis proceeds throughout life, although output decreases with age. Before B cell maturation, during differentiation from CLPs to pro-B cells, recombination activating genes 1 and 2 (Rag 1 and Rag 2) and terminal deoxynucleotidyl transferase (TdT) promotes stepwise V(D)J-rearrangement in the immunoglobulin heavy chain (IgH) locus, that determine the B cell receptor composition [234]. In order to achieve a balance between specificity against pathogens and avoiding autoreactivity, B lymphocytes are screened at a number of checkpoints during lymphopoiesis for their level of autoreactivity [146].

Screening takes place after differentiation of pro-B into pre-B cells. The surrogate light chain (SLC) probes IgH fitness to pair with the immunoglobulin light chain (IgL), and thus a pre– B cell antigen receptor (pre-BCR) complex forms. The IgH chain of the pre-BCR is also checked for autoreactivity. The pre-BCR shuts down expression and activity of the enzyme machinery which catalyzes the rearrangements of the H-chain gene segments, also known as allelic exclusion [231], preventing expression of two IgH chains with two different specificities by the same cell. Thereafter, if the variable region of the IgL chain fits the variable region of the IgH chain, immunoglobulin M (IgM) is displayed as a B-cell receptor (BCR) on immature B cells, with each B lymphocyte expressing one BCR.

Defects in distinguishing between these could lead to autoimmune disease. Normally, when immature B cells migrate to the spleen, they only respond when encountering antigens and thereby proliferate and differentiate into memory B lymphocytes and plasma cells. Apoptosis and anergy can also be induced by antigenic stimulation [197], screening B cells for potential autoreactivity and eliminating them before they become mature. In patients with rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE), reduction of autoreactive B cells can fail at this peripheral checkpoint [273].

1.2.2 Late B cell development

During the stages of B cell differentiation, IgM is first expressed on the cell surface of immature B cells in the bone marrow, from very low to high levels of surface IgM. Immature B cells also express surface markers like CD19, intermediate levels of B220, and they will after maturation express CD21 or CD23, with a few of them also expressing low levels of surface IgD [34]. In order for immature B cells to migrate from the bone marrow to peripheral lymphoid organs such as the spleen, they have to express high levels of IgM on the surface. IgM-expressing B cells transit to the spleen through the terminal branches of central arterioles [9, 10, 133], whereas B cells that are negative for surface IgM do not appear in the spleen. Experiments show that 5–10% of newly generated immature B lymphocytes enter the pool of long-lived mature B lymphocytes [8-10, 37, 70, 132, 197, 198, 200].

Immature B lymphocytes can be distinguished from mature B lymphocytes in the spleen, due to their low expression of B220 and IgD, and their high heat stable antigen (HSA), CD93 and IgM expression [9, 10]. The immature B cell compartment in the spleen has previously been found to be subdivided into two different B cell subsets, transitional 1 (T1) and transitional 2 (T2) B cells [132].

T1 B cells are characterized by high expression of surface IgM, low expression of IgD, and absence of CD21 and CD23, while T2 B cells express high levels of IgM, but are positive for IgD, CD21, and CD23. A third subset of transitional B cells in the spleen has been found, termed transitional 3 (T3) B cells [8]. In the spleen, transitional B lymphocytes complete maturation by developing either into follicular or marginal zone (MZ) B lymphocytes. MZ B cells have BCRs on the surface that bind to blood-borne antigens, like epitopes from bacteria and lipid antigens. In combination with Toll-like receptor (TLR) signals, induced by recognizing pathogen-associated molecular patterns, MZ B cells differentiate into plasma cells, secreting IgM and creating a defense against pathogens in the spleen. For activation of follicular B cells, transportation of antigens into B cell follicles of secondary lymphoid organs is required. Follicular B cells present antigens on Major histocompatibility complex (MHC) class II to T helper cells, upon antigen recognition and activation, in order to receive additional activation signals in form of CD40L and cytokines.

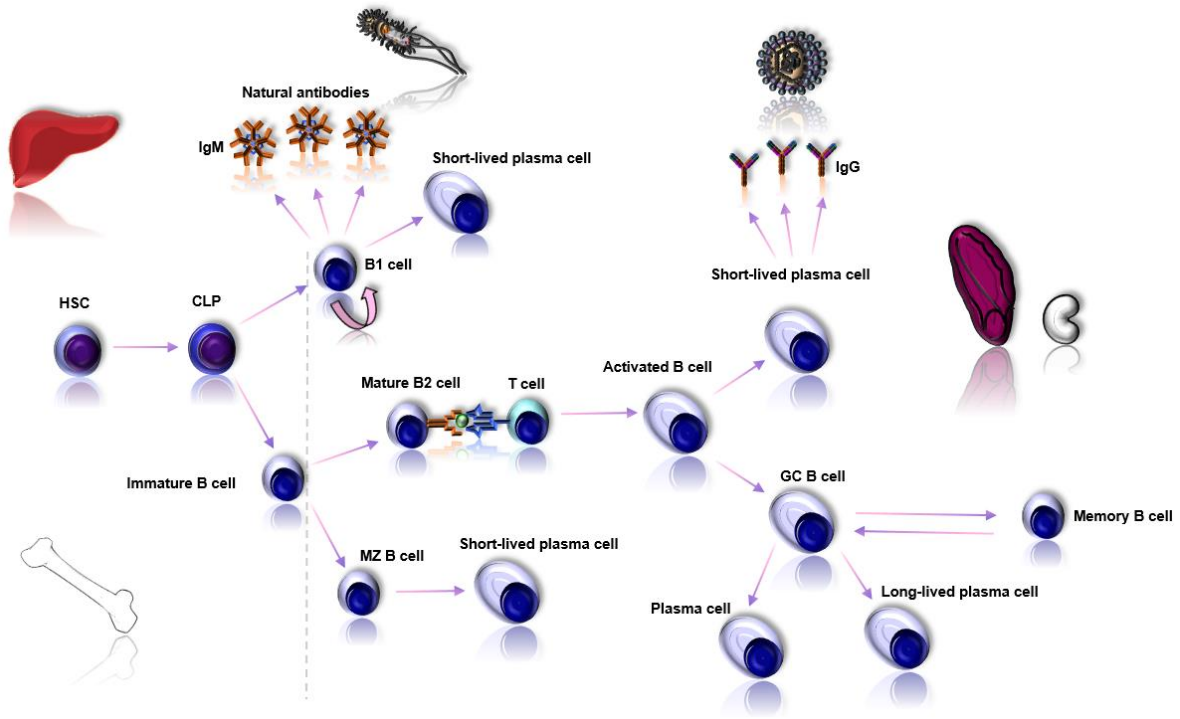

Figure 2. Schematic illustration of B cell development.

Interaction between B and T lymphocytes promotes differentiation of activated follicular B lymphocytes into dividing blasts that form germinal centers (GC). Interaction with GC stromal cells and follicular T helper cells allows GC B cells to undergo immunoglobulin class switch recombination (CSR) to modify the specificities of the BCRs by somatic hypermutation (SHM), and to differentiate into memory B lymphocytes with surface IgG and IgE.

They can also develop into long-lived plasma cells, producing class-switched antibodies. B lymphocytes are the source of the humoral immune system and have been thoroughly studied due to their role in promoting inflammatory responses.

Nevertheless, there exist B cell subsets known to have anti-inflammatory roles. Regulatory B cells (Bregs) have been demonstrated to inhibit inflammatory responses several years ago [151], and since then, other roles for Bregs in different autoimmune and allergic conditions have been found, as well as several diverse mechanisms through which Bregs can reduce inflammation [11, 27, 64, 68, 79, 81, 140-142, 233, 245, 264]. Bregs are immunosuppressive cells supporting immunological tolerance. They suppress inflammation through production of the anti-inflammatory cytokine IL-10, which is one of the most studied mechanisms regarding regulatory B lymphocytes [149, 150, 171]. IL-10 secreting B cells were first characterized in an induced mouse model of contact hypersensitivity, where immune cells, like T cells, provoked inflammation [264]. During the process of an infection, the inflammatory response is crucial for the clearance of pathogens and initiation of protein cascades involved in the healing of wounds [145].

In individuals with chronic inflammation, the immune system is constantly activated, often shown by deficiency in number and function of Bregs at the site of inflammation and in the circulation [158]. Bregs prevent expansion of pathogenic T cells and other pro-inflammatory cells through the production of IL-35, IL-10 and transforming growth factor beta (TGF-β). The usage of genetically modified mice lacking B cells, in particular IL-10-secreting B cells, has shown that defective function and development of Bregs leads to chronic inflammation [68, 258], suggesting Bregs to be future therapeutic targets in cases of autoimmunity, infection and cancer.

1.2.3 Transcription factors important in B cell development

The Ikaros transcription factor family control the specification of HSCs to the lymphoid lineage. Ikaros zinc fingers induce transcription of genes involved in cell cycle regulation, pre- BCR signaling and V(D)J-rearrangement [67]. Experiments in mice have shown that when the gene encoding Ikaros, *Ikzf1*, is deleted, the development of CLPs is impaired. This leads to blockage at early stages of B cell lymphopoiesis, delaying differentiation of thymocytes which are hematopoietic progenitors present in the thymus [75, 271]. Ikaros has also been shown to be a major negative regulator of B1 lymphocyte function and development [136]. There are additional transcription factors with DNA binding domains containing several Zn-fingers, besides members of the Ikaros family, for example BLIMP-1, BCL-6 and GFI, MIZ-1 and CTCF, which regulate critical steps in B cell development [121, 154, 185, 195, 239, 268].

The most crucial steps in early B cell lymphopoiesis are however regulated by the transcription factors E2A, EBF1, FOXO-1 and PAX5, including V(D)J-rearrangement and expression of the pre-BCR. The specification of the CLPs to the B cell lineage is initiated by E2A and EBF1 [159, 168, 218]. In *Ebf1* or *E2A* knockout mice, HSC fail to reach the pro-B cell stage and thus B lymphocytes can not develop [30, 128]. RUNX1 and C-MYC regulate transcription of EBF1 [216, 240], and EBF1 and E2A induce expression of several B cell lineage determining genes, such as *Foxo1* [129, 252, 274], which is needed to activate transcription of *Rag1* and *Rag 2*, required for heavy- and light chain gene rearrangement [58]. PAX5 is involved in B cell lineage commitment of precursor cells, shown in experiments with *Pax5* knockout mice, since pro-B cells are uncommitted in these mice and can develop into many different hematopoietic lineages [41, 56, 166, 167].

1.2.4 The transcription factor Arid3a

ARID3A is one of 15 members of the A+T rich interactive domain (ARID) family of DNA-binding proteins, many of which are shown to have epigenetic regulatory roles [120, 180, 256]. These proteins bind to A+T rich sequences on the DNA and are members of greater chromatin modulatory complexes. Arid3a is one of few members for which a DNA-binding recognition site has been found. Arid3a was first cloned through homology with the orthologue *Dril1* in Drosophila, and later re-identified as a protein related to E2F (E2FBP). Known as a B cell regulator of immunoglobulin heavy chain transcription (BRIGHT) in mouse, this 70 kDa DNA-binding protein has been characterized as a transcription factor that up regulates immunoglobulin heavy chain (IgH) transcription in activated B lymphocytes as part of a greater protein complex that contains the enzyme Bruton's tyrosine kinase (BTK) and the ubiquitously expressed transcription factor II-I [247-249].

The expression of *Arid3a* is limited in adults, however it is widely expressed in embryo and fetus, having important regulatory functions in embryonic stem cell differentiation, highlighting novel roles for *Arid3a* in gene expression [12]. The transcription factor ARID3A is thought to be restricted to cells of the B cell lineage within the immune system, yet others have demonstrated that Arid3a plays important regulatory functions in early hematopoiesis. *Arid3a* is expressed in both murine and human subsets of pre-B cells, transitional B cells, activated B cells, memory B cells and plasma cells. Nevertheless, most of the resting, naïve and mature peripheral B cells do not express *Arid3a* [165, 250]. Innate-like B cells named B1 cells, predominantly found in the pleural and peritoneal cavities, have in previous studies been shown to express lower levels of ARID3A [36, 152, 164]. Hence, the expression of *Arid3a* is thought to be strictly regulated at the level of transcription throughout B lymphocyte development.

1.3 DISEASE MODELS

1.3.1 Rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease, characterized by synovitis, which leads to destruction of the cartilage and bones. The pathogenesis of RA is complex and involves both genetic and environmental factors initiating inflammatory responses resulting in swelling and destruction of the tissue. Studies have shown that a high-risk genetic background together with environmental stimuli and epigenetic changes result in a cascade of inflammatory events causing activation of macrophages by autoreactive T cells, which leads to the release of pro-inflammatory cytokines like interleukin 1,6 and 17 (IL-1, IL- 6, IL-17) and tumor necrosis factor α (TNF- α). [72, 108, 225].

There are several immunotherapies that have shown to be successful in disease management, targeting cytokines, their receptors or other signaling components downstream [72], however the events that actually cause the recruitment of autoreactive lymphocytes is still under exploration. Recently, reactivity to citrullinated antigens in the initial pathogenesis has been elucidated in several autoimmune diseases, including RA, proposing citrullination to be of great importance in the process of epitope spreading and the emergence of auto-epitopes [35, 65, 155, 221]. Detection of anti-citrullinated protein (ACP) antibodies is used for the diagnosis of RA, is a highly specific method and can be used even before the clinical disease onset [42].

Animal models are essential for understanding the pathogenesis and induction of autoimmune disease and for being able to develop therapeutic interventions that can detain disease progression or even effectively treat the disease. Collagen-induced arthritis (CIA) is the most common and well-studied rodent model of human RA. CIA can be used for both rats and mice, and is induced by immunization of Type II collagen (CII), which is the main constituent collagen form in articular cartilage, previously identified in RA patients [48, 104, 229, 236]. After disease onset, rodents develop a monophasic erosive polyarthritis, characterized by swelling of the joints in toes and wrists. Several features of RA is present in mouse CIA as well, like anti-citrullinated peptide antibodies and rheumatoid factors, making this animal model suitable for studies of rheumatoid arthritis [215].

1.3.2 Multiple sclerosis

Multiple sclerosis is a degenerative and demyelinating autoimmune disease of the central nervous system (CNS), involving mechanisms of both the innate and adaptive immune system. MS has a highly complex, heterogeneous pathogenesis, believed to result from interactions of polymorphic genes, epigenetic regulation and environmental factors such as smoking, commensal microbiota, virus infections, demographics and Vitamin D deficiency [22, 24, 49, 203, 208, 210, 235]. Genome-wide association studies (GWAS) have revolutionized the field of autoimmune disease [100], and more than 100 single nucleotide polymorphisms (SNPs) have been associated with MS [211]. This polygenic disease can have several courses and different progression rates.

The most frequent phenotype is relapsing-remitting MS (RRMS), which covers around 85% of newly diagnosed patients, characterized by phases of deterioration, followed by spontaneous remission. Initial RRMS most often develops into secondary progressive MS (SPMS), described as a progressive worsening of neurologic function without any remissions. About 15% of newly diagnosed patients develop clinical primary progressive MS (PPMS), without any preceding relapses or remissions [80, 114, 170]. There exists several different animal models of MS, however the most studied and best understood is the rodent model of Experimental Autoimmune Encephalomyelitis (EAE). Several therapies have been developed and tested using this rodent model of disease, such as Natalizumab (Tysabri), before going further into clinical trials [269].

EAE, first time induced over more than 60 years ago, is an acute or chronic relapsing disease characterized by demyelination of axonal tracks throughout the central nervous system, leading to weakness and paralysis of the hind limb legs in rodents, followed by spontaneous remission[173]. In EAE, monocytes and T cells infiltrate the CNS, causing local inflammatory responses with proteins expressed by myelin-producing oligodendrocytes as targets. This then leads to demyelination of the axons and impaired axonal conduction. Currently, there are several pathophysiological types of EAE with different patterns of presentation, depending on which peptide or protein and which animal model that is being used. This disease model has a clinical course, which is characterized by a prodromal period of 10-15 days, followed by weight loss and paralysis of the tail and hind limbs, and finally proceeding to the other limbs. In some animal models, the disease follows a course similar to relapsing-remitting MS, which gives opportunities for immunomodulatory and mechanistic studies.

1.3.3 Atherosclerosis

Atherosclerosis is a chronic inflammatory disease of the arteries, characterized by deposition and trapping of low-density lipoprotein (LDL) in the artery walls, and is the underlying cause of many cardiovascular disorders, such as stroke, myocardial infarctions and peripheral vascular disease. Both the innate and adaptive immunological arms are involved and the response is often due to hyperlipidemia [13 77, 204]. In atherosclerosis, LDL is modified by a variety of enzymatic and non-enzymatic alterations, leading to cascades of inflammatory responses followed by recruitment of immune cells like T cells and macrophages [227]. Both cells of the immune system and cells of the vessel walls participate in atherogenesis, heavily influenced by genetics, lifestyle and diet, hemodynamics of the blood flow in the arteries and by plasma lipoproteins.

A large number of inbred mouse strains have been crossed into the background of genetically modified atherosclerotic models, resulting in diverse susceptibility to develop atherosclerosis, and leading to the identification of genes involved in the determination of resistance and sensitivity to atherosclerosis[220, 232, 266]. Mouse models of atherosclerosis are dependent on increasing blood plasma levels of LDL and very low-density lipoprotein (VLDL), and thus these murine atherosclerotic models are often based on genetic ablation of apolipoprotein E (ApoE) or the LDL-receptor.

B-cell immune responses have been identified to be involved in atherosclerosis and coronary heart disease (CHD) [106]. Functional roles of B cells in experimental atherosclerosis have been demonstrated by splenectomized *Apoe* knockout mice, showing that these mice develop aggravated atherosclerosis. Nevertheless, this effect could be reversed by transfer of educated splenic B lymphocytes. Transferring educated splenic B lymphocytes from older *Apoe* knockout mice both rescued the pro-atherogenic effect and reduced the size of lesions [33]. Regulatory B cells (Bregs) have a different surface marker expression compared to other B cells and have been shown to be involved in autoimmunity, e.g. by the secretion of interleukin-10 (IL-10) or direct interaction with pathogenic T cells [265]. The role of Bregs in atherogenesis still remains to be characterized.

Even though studies of currently existing atherosclerotic mouse models noticeably have led to a better understanding of atherogenesis, many features of present mouse models differ from the human disease [23, 53, 147], highlighting the importance of finding new murine models, better applicable to human atherosclerosis and the underlying mechanisms.

2 METHODOLOGICAL CONSIDERATIONS

2.1 ANIMAL MODELS

In our studies, we used several different murine models for investigating various autoimmune disorders, by the usage of the Cre/lox system. In this system, the exon of interest is flanked by loxp sites and driven under a promoter. When this promoter is transcribed, the enzyme Cre is produced, leading to cleavage at the loxp sites, thus deleting the flanked exon in all cells that express the promoter. In the first study, we obtained a conditional allele of *Arid3a* and deleted this allele in all cells expressing *Mb1*, also known as *Cd79a*, by using an Mb1-Cre line. *Mb1* is known to be unique for B cells, and is expressed from early progenitors, throughout B cell development and differentiation [101]. Hence the *Arid3a* was removed in all stages and subsets of B lymphocytes.

In the second and third study, we were interested in studying the germinal center reaction in various inflammatory disorders. We deleted *Pax5*, using Cd23-Cre and Aid-Cre. PAX5 is essential for B cell function, so loss of this transcription factor disrupts B cell function. [144]. CD23 is expressed on mature B cells and AID is characteristic for germinal center B cells. These deletions resulted in two conditional knockout models for mature and germinal center B cells.

In the fourth study, we created an inducible mouse model of atherosclerosis. APOE is a protein involved in the metabolism of body lipids, and is found on LDL and VLDL particles where it functions as a ligand for the LDL receptor. Deleting the gene encoding for this protein results in hypercholesterolemia and subsequently atherosclerosis. To investigate the development of these lesions and the implication of the immune system, we used the Cre/lox system, which was activated in mice by administration of tamoxifen, thereby deleting the gene in all cells.

2.2 GENOTYPING WITH POLYMERASE CHAIN REACTION

Genotyping is a method which we frequently used for distinguishing experimental mice from littermate controls, as well as for putting together new breeding pairs of mice. By extracting DNA from a small ear biopsy, we could with the help of specific primers and polymerase chain reaction (PCR) amplify a sequence of interest, determine its length through gel electrophoresis, and hence get information about the genotype.

2.3 FLOW CYTOMETRY

For the characterization of cell subsets and populations within an organ, we used flow cytometry and phenotypic markers. We stained single cell suspensions with carefully chosen fluorescently labeled antibodies, and expression of target antigens can define population of cells. Thereafter, we acquired these suspensions in a flow cytometer, where different lasers inside the machine detected the fluorescently labeled markers, thereby quantifying the surface receptors, while also distinguishing cells by size and granularity.

This method is highly advanced and gives the opportunity to describe a wide range of cell populations, however it is important to work properly and wash the suspension thoroughly, since overstaining could give inaccurate results and cross-contamination easily occurs. It is also necessary to use unstained and single stained-controls for each marker, in order to compensate for the spill of fluorescence between the markers, when analyzing the results in a proper software. For analysis of flow cytometry-data, we used the software FlowJo.

2.4 CELL SORTING AND DELETION ANALYSIS

In order to confirm the deletion of the flanked exon in our first study, we sorted B cells with the help of Fluorescence-activated cell sorting (FACS) and specific markers. We then extracted RNA from the sorted cells, made cDNA, amplified the specific sequence of interest with PCR and visualized the sequence of interest with gel electrophoresis. With this method, we could confirm that the gene was present in control littermates and deleted in experimental mice, however a limitation with this method is that it does not provide any information on a protein level.

2.5 WESTERN BLOT

For confirming the loss of the transcription factor we investigated in the first study, we used western blot. This method demonstrates, based on size, whether a protein is present or removed in a certain tissue or cell population. We tried various commercial antibodies, which in theory should bind to the protein we studied. Still, none of these antibodies bound to the target protein in a specific manner, although several different protocols were assessed.

We confirmed that the protein extraction and our western blot technique worked, by visualizing Actin, one of the most abundant proteins in eukaryotic cells. Indeed, the western blot confirmed that the antibodies for Arid3a bound unspecific. Hence, we targeted the *Arid3a* locus with sequencing and found that a loss of the flanked exon 4, a part of the DNA-binding domain of our transcription factor, resulted in an allele out of frame and a non-functioning protein.

In study IV however, we successfully could confirm the deletion of APOE, by using an antibody that showed correct specificity towards our protein of interest.

2.6 ENZYME-LINKED IMMUNOSORBENT ASSAY

Enzyme-linked immunosorbent assay (ELISA) is a highly sensitive method for detection of specific proteins. We used this method in all of our studies in order to measure the levels of a wide range of antibodies in blood plasma. Besides measuring the levels of immunoglobulins in study I, we also measured antibody responses to phosphorylcholine after intraperitoneal immunization. In study II, we used ELISA for measuring antibodies against CII and in study III we looked at antibodies against MOG protein and MOG_{79-96} peptide. ELISA could also be used for measuring cytokines like IL-10, since it gives a very high accuracy and can detect very low levels of proteins.

2.7 INDUCTION AND EVALUATION OF COLLAGEN-INDUCED ARTHRITIS

For the induction of experimental arthritis in the mice used in the second study, we injected male mice with CII in complete Freund's adjuvant intradermal at the tail base. 35 days later, the mice were given a second injection of CII in incomplete Freund's adjuvant to boost the development of disease. The complete form of the adjuvant contained inactivated mycobacteria, whereas the incomplete form lacked the bacterial components and only consisted of water in oil-emulsion.

The adjuvant is designed to continuously release the antigens necessary for stimulating an immune response, however the disadvantage is that it causes local irritation and wounds at the site of injection. The mycobacteria in the initial injection attracts phagocytic cells to the injection site, enhancing the immune reactions. However, due to the previously mentioned side-effects, the incomplete form of the adjuvant was used for boosting.

For daily evaluation of the disease development after onset, we used a certain scoring system, where each inflamed toe or knuckle was given one point, and swollen ankles or wrists 5 points, resulting in a maximum of 60 points per animal. This scoring system is a valuable tool for following the disease course, while emphasis should be put on having a blinded evaluator and scoring in a consistent manner.

2.8 INDUCTION AND EVALUATION OF EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

In order to induce EAE during study III, we injected female mice intradermal at the tail base with either MOG_{79-96} peptide or the full-length MOG protein, both in incomplete Freund's adjuvant. To enhance the effect of disease development, we gave the mice an intraperitoneal injection of *Bordetella* pertussis toxin on the same day of MOG administration, and another injection two days later. Pertussis toxin is widely used to facilitate the induction of EAE and is considered to open up the blood-brain barrier, thereby promoting pathogenic T lymphocytes to migrate into the central nervous system [102]. Other suggested biological effects of this toxin are enhancement of cytokine secretion by T cells and induction of lymphocyte proliferation [102].

For daily disease evaluation, we used a specific scoring system, created for this autoimmune disease model, based on balance, weakness and paralysis of tail and limbs. Similar to the former mentioned evaluation of CIA in study II, it is of high importance to be blinded and keep consistency when using this scoring system. Additionally, we weighed the mice daily and kept track of weight loss, which correlated with disease severity.

2.9 IRRADIATION AND BONE MARROW TRANSPLANTATION

Numerous bone marrow transplants were performed in study IV. The recipient mice, belonging to an inducible mouse model of atherosclerosis, were irradiated twice with three hours apart, in order to deplete their immune system, but preserve their gut flora. They were thereafter injected in the tail vein with bone marrow cells from mice deficient in mature or germinal center B lymphocytes. This method reconstituted the immune system of the irradiated recipient mice, through bone marrow progenitor cells of donor mice. Since the donor mice were deficient in mature or germinal center B cells, the same deficiencies were obtained by the recipient mice.

2.10 CELL ISOLATION FROM AORTA

In order to investigate and characterize the cell populations present in the mouse aorta in the fourth study, we dissected aortas from experimental mice and littermate controls, and digested them by using specific enzymes. After a sufficient time of digestion, we stained the single cell suspensions with specifically chosen fluorescently labeled markers and acquired them using flow cytometry, as previously described.

3 RESULTS AND DISCUSSION

3.1 STUDY I: A ROLE OF THE TRANSCRIPTION FACTOR ARID3A IN MOUSE B2 CELL EXPANSION AND PERITONEAL B1A GENERATION

B1a cells, also named $CD5^+$ B cells, are self-renewing cells, persisting throughout the whole adult life [94], producing natural autoantibodies [92, 93]. They are derived from the fetal liver, as well as the bone marrow in the adult mouse, and are abundant in the peritoneal cavity of adults [90, 91]. Autoantibodies, reactive to numerous plasma antigens and both surface and intracellular structures, are found in healthy individuals and germ-free mice [148]. These antibodies are multi-reactive, do not undergo affinity maturation, and are believed to be involved in several physiological events such as homeostasis, immune regulation, resistance to infections and modulation of molecules [14, 15], but are also thought to be involved in autoimmune disease like Type 1 diabetes mellitus [190] and SLE [83].

Antibodies associated with B1 cells can cross-react with self-antigens, leading to autoimmune diseases, but B1 lymphocytes are also known to play important protective roles in infectious diseases. Nevertheless, B1 cells have shown to be involved in cancer, such as B cell acute lymphoblastic leukemia (B-ALL) [153] and chronic lymphoid leukemia (CLL) [90]. Currently, there exists various B1 deficient strains, however they have defects in other B cell subsets as well, such as in the B1b or B2 subsets [181 62, 187]. Particularly mice with deletion of *IκBNS* showed interesting effects of being deficient in B1a cells, but still producing low numbers of B1b cells [181]. Thus, we were highly interested in creating a murine model lacking only B1a cells, and characterizing the effects of this deficiency.

The ARID family consists of 15 DNA-binding members, involved in proliferation, differentiation and regulation of chromatin accessibility. Some of these ARID family members have been shown to be involved in various cancers [32] and in the autoimmune disease SLE [244]. Arid3a, referred to as Bright in mice, is known to activate transcription of IgH and has been proposed to be a proto-oncogene. This protein has previously also been shown to be a key transcription factor, critically regulating the B1 versus B2 fate in development of B lymphopoiesis [127]. Arid3a is known to alter IgH V gene expression [99, 250] , regulate the BCR signaling [213, 251] and play important roles in transcriptional activation and cell growth [206]. Arid3a has been identified as a key target of the microRNA *Let-7*, highly expressed in the hematopoietic system [272]. Ectopic expression of *Let-7* induced development of B1 cells from adult pro-B cells and silencing by knockdown inhibited development of B1 lymphocytes in fetal pro-B cells [277].

Let-7 and the RNA-binding protein LIN28B have been suggested to play critical roles in specifying the B1 versus B2 cell lineage [272]. According to the Immunological Genome Project (ImmGen) database [98], *Arid3a* is expressed in B lymphocytes, particularly in the early progenitors. We were therefore interested in conditionally deleting the gene encoding for this transcription factor and investigating the effects of this on B lymphocyte development and B cell subsets, like the B1a subset.

Deletion of *Arid3a* has previously been shown to result in embryonic lethality, with less than 1% of the mice surviving to adulthood [246]. These rare survivors showed multiple severe defects in both hematopoietic stem cells and erythropoiesis [246]. To circumvent embryonal lethality, we obtained a conditional allele of *Arid3a* and assessed the function of ARID3A in early and late B cell lymphopoiesis using the Mb1-Cre line to determine any possible developmental defect in the B cell lineage due to loss of this transcription factor. In our construct, exon 4, the DNA binding domain of Arid3a [99], was flanked by loxp sites. When crossed with the Mb1-Cre line, known to be specific for B lymphocytes from early stages and throughout B cell development and differentiation [101], exon 4 would be removed by Cre recombinase, resulting in an allele out of frame and a non-functioning protein.

We confirmed deletion of the allele in B cells sorted from both bone marrow and peritoneal cavity, characterized various subsets of B lymphocytes and found that the absolute cell numbers were affected in almost all B cell subsets in bone marrow and spleen, due to loss of this transcription factor. All investigated stages of B2 cells, from early progenitors in the bone marrow to late mature stages found in the spleen, were greatly expanded, suggesting a possible function for Arid3a in leukemia, for example B-ALL. In the peritoneal cavity, the previously mentioned B1a cells were strongly reduced, proposing Arid3a to be important for the production of B1a cells or for the migration of B1a cells to the peritoneal cavity.

Even though we could confirm that the *Arid3a* allele was deleted both in bone marrow and peritoneal cavity by Reverse transcription polymerase chain reaction (RT-PCR), we were not able to measure the protein levels of ARID3A via Western blot, although having used several diverse antibodies. We hypothesized that this was due to unspecific or no binding of these antibodies, nevertheless, we could not rule out that a truncated protein was not produced. However, since previous studies have shown that conditional deletion of exon 4 in the highly related *Arid3b* led to loss of the protein [123], we proposed a likewise outcome for our protein. Furthermore, following the loss of exon 4, an out-of-frame transcript was detected, encoding for a nonsense protein.

Retrieving information from the ImmGen database [98], we saw that *Arid3a* also is expressed in granulocytes, particularly neutrophils from the bone marrow, which made us curious to interrogate the conditional loss of this allele in this type of immune cells and compare it to the effects we saw on B cells. Thus, we created a lineage-specific deletion of *Arid3*a, via S100A8- Cre, formerly shown to be neutrophil-specific [161, 241]. Looking at various different immune cells like neutrophils, monocytes and macrophages, we saw no significant effects upon loss of this gene, supporting the hypothesis that Arid3a is B lineage specific and important in several diverse B cell subsets. In order to further investigate the specificity of Arid3a and interrogate a possible implication in disease like cancer, we created an additional Arid3a murine model, crossed with Vav1-Cre, previously shown to be specific for hematopoietic stem cells [74, 278].

Analysis of several different B cell subsets in mice lacking *Arid3a* in the hematopoietic stem cells showed no significant differences, indicating no direct function for Arid3a in HSCs and early progenitors, in contrast to the previously mentioned study where *Arid3a* had been deleted [246]. It is unclear whether the effect they saw upon loss of *Arid3a* in hematopoietic stem cells was dependent on the closely related *Arid3b*, however conditionally deleting *Arid3b* resulted in unperturbed HSC populations [123], suggesting that HSC development is independent of *Arid3b*. The latter study showed additional results, indicating both ARID3A and ARID3B transcription factors to be required for B cell development [123].

Since Arid3b is closely related to Arid3a [256] and the formerly mentioned study showed that both of these transcription factors are important for B cell lymphopoiesis [123], we hypothesized that the loss of *Arid3a* might be compensated by *Arid3b*. Nevertheless, it has formerly been demonstrated that this closely related paralogue, also named *Bdp*, is not upregulated due to loss of *Arid3a* [246], and thus it is unlikely that paralogous redundancy occurs in our murine model. These results together with our previous results suggest important functions for Arid3a in B lymphocytes and propose Arid3a to be restricted to the B cell lineage. According to the ImmGen database [98], *Arid3a* is not highly expressed in hematopoietic stem cells of mice, however the expression in human hematopoietic stem cells is higher, and thus one should not rule out that Arid3a could be important for hematopoietic stem cells of humans.

A previous study suggests this transcription factor to induce autoimmunity and proposes an *Arid3a* transgenic model that could be used for future analyses of B lymphocyte autoreactivity [217]. In that study *Arid3a* was constitutively expressed in all B lineage cells, leading to an increased total amount of B cells in the bone marrow, however not in the individual subpopulations.

The transitional B cell numbers in the spleen were expanded in that murine model, as well as the IgG levels in serum. Non-pathogenic autoantibodies against phosphorylcholine (PC) are produced by B1 cells and occur naturally. The anti-PC responses in the mentioned study were enhanced, however responses to other foreign proteins did not occur. These results from overexpression of *Arid3a* are in concordance with our results after conditionally deleting this gene, except the fact that they saw no response to other foreign antigens, since we have seen significant differences of antibody levels in serum from our murine model after immunization with NP-Keyhole Limpet Hemocyanin (NP-KLH).

Former studies have revealed that antibodies against PC are anti- inflammatory, playing an important protective role in cardiovascular disease (CVD) [69]. It has been proposed that IgM antibodies against PC could be protection markers for CVD, where low levels of IgM anti-PC would be a marker for increased risk of developing CVD [71]. These results indicate a possible implication for Arid3a in atherosclerosis and CVD, since the levels of antibodies against PC were greatly reduced in both naïve and immunized mice in our conditional model.

An interesting aspect of overexpressing *Arid3a* was the presence of anti-nuclear antibodies (ANAs), used to characterize autoimmune diseases like SLE, in the serum of young transgenic mice [217]. Another study, with numerous SLE patients involved, correlated dramatically increased numbers of Arid3a$^+$ B cells with increased disease activity, suggesting ARID3A as a potential marker for B cells correlated SLE [244]. These results together with our findings highlight the importance of further investigating the function of Arid3a and propose our model to be useful for diseases where natural antibodies are implicated.

3.2 STUDY II: GERMINAL CENTER B CELLS ARE ESSENTIAL FOR COLLAGEN-INDUCED ARTHRITIS

Rheumatoid arthritis (RA) is a chronic inflammatory, autoimmune disease, characterized by swelling of the joints, persistent synovitis, autoantibodies like rheumatoid factor and anti-citrullinated protein antibodies (ACPAs), and destruction of the cartilage in the joints, resulting in severe disability. RA affects 0.5-1% of the adult population [97], however the etiology of the disease is not fully understood. Nevertheless, it is known that both B and T cells are of pathogenic importance in the disease [143], which highlights the significance of investigating the function and implication of the adaptive immune system in RA.

Antibody specificities occurring in collagen- induced arthritis (CIA), a disease model commonly used in studies of RA, where rodents develop arthritis after immunization with Type II collagen (CII) [50, 236], have been demonstrated in clinical subsets of RA [31], emphasizing the relevance of interrogating the disease course of CIA and the implication of antibodies to better understand experimental arthritis. B lymphocytes secreting antibodies to CII are found early in the disease and IgG has been shown to be the main subtype [182, 229]. It has also been demonstrated that the B lymphocyte response to CII in RA patients is associated with HLA-DR4, hence reflecting a CII-specific activation of T lymphocytes [201, 205]. B lymphocytes are known to be of high importance for experimental arthritis, however which B cell subset is responsible for disease development and progression has been unknown.

Histological observations in patients with RA have elucidated the presence of germinal centers (GCs) in inflamed joints [214, 228], and similar results have been demonstrated in the joints and secondary lymphoid tissues of mice [84]. Other studies have directly suggested the formation and function of GCs as important contributors to RA [257], endorsing the importance of our study. However, antigen-collagen antibodies are of a germline configuration. It is not clear what function GC formation may perform in the pathogenesis of CIA.

CIA is a frequently used and accepted disease model of RA, nevertheless there are obvious differences between the human disease and the murine model [117, 259]. One of these differences is the reversed gender susceptibility. While female predominance is characteristic for RA [52], the incidence for male mice to develop CIA is greater than for female mice. Studies have proposed a protective function of estrogen in female mice [110], yet others have demonstrated both anti-inflammatory and pro-inflammatory functions for estrogen [223].

Why autoimmune disease like RA has a high prevalence in women however, remains unclear [66], highlighting the complexity of this disease and the immuno-modulating role of estrogen. Since most published CIA experiments have been performed on male mice and our aim was to investigate the function of B cells and possibly clarify which subset could responsible for disease development and progression, we decided to use male mice only.

The disease is clearly associated to the MHC-region, and therefore we used B6NQ mice expressing the H-2^q haplotype, previously shown to sufficiently develop disease [226, 260, 261]. This H-2^q haplotype is required for the presentation of the immunodominant T cell epitope from collagen. We first verified that the CII we were using functioned as desired and that our models were susceptible to CIA. Our results were in agreement with formerly presented data, both the disease development and the progression, as well as the antibody titers [226].

We subsequently used two different conditional gene knockouts to interrogate the disease course of CIA, and to further clarify the function of B cells and antibody secretion in disease. These two murine models were deficient in either the mature or the GC subsets of B lymphocytes, the latter one important for antibody production.

In the first murine model, follicular and marginal zone B cells were depleted, as well as memory B cells, while the second mouse model lacked only the GC B cell subset. Both models showed similar results in cell number and antibody levels compared to wildtype litter mates, since transitional B cells develop into follicular B cells and then further into GC B cells. When deleting in the GC subset, the mouse will lack only germinal center B lymphocytes, however when deleting in the late transitional stages, all subsets that transitional B cells could differentiate into will be depleted, including the GC B cell subset. Indeed, our data showed that both models lacked GC B cells, as well as had no or very low production of IgG and antibodies against Type II collagen.

These GC-deficient mice had on the other hand normal levels of natural antibodies, since the main subset secreting IgM is B1a cells, predominantly found in the peritoneal and pleural cavities. The most interesting finding however, was that mice lacking G B lymphocytes were fully protected against experimental arthritis, clarifying the role of GC B cells in CIA and proposing important functions for GC B cells in RA. Mice that are B cell-deficient and thus lacking antibodies have previously been shown to be protected against CIA [226], in line with the results we demonstrated in both of our murine models.

Early treatment of the disease in patients is required to prevent and reduce injury of the joints and bone erosion. Currently, several different treatments exist, such as disease-modifying anti-rheumatic drugs (DMARDs) [176], TNF inhibitors [139] and newer biological treatments like B cell depleting agents [16, 219]. These therapies slow down disease progression, reduce synovitis and systemic inflammation, subsequently relieving joint pain and tenderness. However not all patients respond sufficiently to these treatments and sometimes they have serious side effects, making the search for newer and more refined therapies important. Various antibody therapies targeting B lymphocytes have been demonstrated, such as Rituximab [43, 61, 63], Ofatumumab [177, 230] and Ocrelizumab [73, 222], which bind to membrane-bound CD20 of B lymphocytes, depleting them through various mechanisms like antibody- or complement-dependent cellular toxicity and induction of apoptosis. However, pro-B and pre- B cells, as well as plasma cells, do not express CD20, and thus anti-CD20 therapies do not prevent regeneration of B cells from precursors, nor do they directly interfere with antibody production.

Our study demonstrates that germinal center B lymphocytes are essential for experimental arthritis and highlights the importance of further investigating this antibody-producing subset in autoimmune disease. Although the role of B lymphocytes and the implication of antibodies in clinical arthritis is likely to be more complicated, targeting germinal centers in RA could help refining existing B lymphocyte depleting therapies or possibly aid in creating newer, more defined therapies.

3.3 STUDY III: ANTIGEN-DEPENDENT FUNCTIONS FOR GERMINAL CENTERS IN EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

B lymphocytes are essential for both the adaptive and the humoral immunity, producing antibodies to help eliminate foreign pathogens and generating a memory, responsible for an accelerated and more specific antibody-mediated immune response upon re-exposure to the same pathogen [3, 125]. It has also been revealed that B cells contribute to regulation and pathogenesis of autoimmune disease like multiple sclerosis (MS) [193], however the underlying mechanisms are still under exploration, since B lymphocytes have numerous functions in immune responses.

MS is a demyelinating inflammatory disease, considered to be mediated by T lymphocytes, resulting in inflammatory lesions, cell infiltration and axonal damage in the central nervous system (CNS). To study the disease course and pathogenesis of autoimmune disease such as MS, we adopted a well-studied model of inflammatory brain-demyelinating disorders, referred to as Experimental autoimmune encephalomyelitis (EAE) [20, 131, 179]. We investigated the function of B cells and antibodies in this disease by immunizing mice lacking germinal centers (GCs) with either myelin oligodendrocyte glycoprotein (MOG) or MOG_{79-96} peptide, both known to be potent encephalitogens for EAE induction [2]. The disease onset and progression are in concordance with an important study, where the susceptibility of different MHC classes to MOG was investigated using several congenic mouse models and numerous of MOG peptides [2]. Additionally, others have demonstrated a similar disease course, in agreement with what we demonstrated in our EAE studies [26].

Our results show that the loss of GC B cells alters the disease in an antigen-dependent manner, proposing that the GC response could protect as well as promote EAE, highlighting the importance of GC formation in disease development and progression. The disease model of EAE has both similarities with and differences from the human disease, such as the fact that multiple sclerosis affects women three times more than men [47].

Likewise, mainly female mice are susceptible to EAE [47, 191]. For that reason, we used only female mice in our study. Due to that MS and EAE are both considered to be T cell mediated diseases [55], we were interested in looking at the T cell subsets and clarify whether those were affected in our disease model as well.

Interestingly, in contrast to former studies [55], we did not see any differences in various T cell subsets between experimental mice and littermate controls, except a significant decrease in regulatory T cells in the inguinal lymph nodes. Since our conditional mouse model lacked GC B cells, and the germinal centers are the key sites of class-switched antibody production [55], the production of IgG and antibodies against both MOG protein and MOG_{79-96} peptide was depleted. The levels of natural antibodies however, were almost unchanged, since this subset of antibodies are mainly produced by B1 cells, predominantly found in the pleural and peritoneal cavities [88, 209]. The involvement of B cells in EAE is still under exploration. Some studies have suggested B cells to be responsible for disease development [186], while others have demonstrated protective roles for B cells [264].

Additionally, other groups have demonstrated that the roles of B cells in EAE is subset-dependent [140, 141]. We showed, with the help of a germinal center-deficient mouse model, that GC B cells can both be pathogenic and protective, depending on antigen used for induction. This suggests a regulatory role for B cells in EAE, in agreement with previous studies [68, 141]. The mechanisms behind these findings remain unknown and need to be further investigated, however it is likely that antibodies are involved, since the GCs are responsible for production of IgG and other class-switched antibodies. Oligoclonal IgG is common in MS patients [29, 115], and levels of IgG in central spinal fluid can be directly correlated to disease progression [255].

We could propose that disease progression might be independent of antibodies, since B cell-depleting therapies result in improvement of autoimmune disease. It has been shown in both mouse and man that B lymphocytes secrete elevated levels of the pro-inflammatory cytokine IL-6 during disease, and that IL-6 deficiency results in less severe disease [18]. Nevertheless, our findings demonstrate that it is improbable that the disease course is independent of antibodies, and more likely that the mechanisms are highly complex.

Whether EAE is a good model for studying MS is controversial. While EAE is the most commonly used disease model for human inflammatory demyelinating disease [2, 47] , others propose that this disease model is unlikely to be a useful model for demyelinating disease, but rather a model for human acute disseminated encephalomyelitis or acute hemorrhagic leukoencephalitis [21].

EAE resembles the symptoms and pathology of MS in several ways, and although this animal model differs from the human disease in many aspects, much of our understanding regarding the inflammatory and autoimmune mechanisms is due to this disease model. At present, several different therapeutics for treating MS exists, like general lymphocyte-depleting therapies [109], more specific B cell-depleting therapies [89] and monoclonal antibodies targeting certain structures found on certain cell types [25]. Several MS therapies have been tested on the EAE disease model [196], highlighting its validity as a model for MS and for developing more refined treatments.

3.4 STUDY IV: ACUTE LOSS OF APOLIPOPROTEIN E TRIGGERS AN AUTOIMMUNE RESPONSE THAT ACCELERATES ATHEROSCLEROSIS

Atherosclerosis is a chronic inflammatory disorder, where both cells of the immune system and cells of the vessel walls are involved. Atherosclerosis is the underlying cause of most cardiovascular diseases (CVD), leading to stroke and myocardial infarctions [86]. The atherogenic process affects both the innate and adaptive arms of immunity, often activated due to hyperlipidemia, and resulting in the development of atherosclerotic plaques that can trigger the formation of occlusive thrombi [13, 77, 204].

Except pro-inflammatory lipoproteins and hemodynamics of the blood flow in the arteries, an important factor that can influence atherogenesis is, heritability [212]. GWAS have identified 58 regions in the genome, where SNPs are associated with increased risk of developing coronary artery disease [46, 163]. Since atherosclerosis is a leading cause of CVD worldwide, we were interested in investigating this disorder and possible risk factors with the help of a novel atherosclerotic mouse model. Numerous animal models for studying atherogenesis exist, however none of these are ideal, due to limitations in disease development and large differences from the human disorder like heart rate, total plasma cholesterol, sites of atherosclerosis development and the time to develop atherosclerotic lesions [76].

Most importantly, humans develop atherosclerosis in the adult stage of life, yet the most frequently used mouse models of atherosclerosis, *Apoe* $^{-/-}$ or *Ldlr* $^{-/-}$ [262], are deletions of genes during early embryogenesis [220, 266]. Therefore we aimed at creating an inducible atherogenic mouse model, which we could use for studying the early immune responses and the development of atherosclerotic plaques, better resembling events in the human disease before and at disease onset.

Our findings showed that inducing deletion of *Apoe* in the adult stage of the mouse triggers autoimmune responses, resulting in acute hypercholesterolemia and accelerating the formation of atherosclerotic lesions. We could verify the loss of apolipoprotein E and showed that the cholesterol levels raised significantly in our experimental group, compared to the littermate controls, just a few days after gene deletion. The cholesterol levels continued to raise and 140 days after disease induction, the levels were almost 4-fold higher in experimental mice, than in the control group, in contrast to the triglyceride levels, which remained unaltered between the groups.

We also quantified atherosclerotic lesions in the aortas and demonstrated that acute loss of *Apoe* resulted in development of plaques in experimental mice, while littermate controls developed none. These results are in agreement with previously revealed data, where Apoe-deficient transgenic mice demonstrated severe hypercholesterolemia and developed atherosclerotic lesions, similar to those in humans [157]. It is known that B cells are implicated in the development of atherosclerosis, however the underlying mechanisms of atheromodulation is still unexplored. We further investigated the spleen and lymph nodes, as well as digested aortas for analysis, in our inducible mouse model, and found no differences in the transitional, follicular or marginal zone B cell subsets, neither did we see any general cell expansions of B cells, T cells and myeloid subsets upon acute loss of *Apoe*.

Looking at the T helper cells and regulatory T cells, we could confirm an increase in the experimental mice, but one of the most interesting findings was the large increase in cell numbers of germinal center (GC) B cells, the subset which is responsible for producing class-switched antibodies, early after the deletion of *Apoe*. These results are in accordance with findings reported in a previous study that proposes a role for regulatory T cells in controlling the germinal center reaction in peripheral lymphoid organs and a pro-atherogenic function for germinal centers [40].

In line with already mentioned results, we saw increased levels the cytokine interferon gamma (IFN-γ), known to be pro-atherogenic [253], as well as increased levels of IL-4 and IL-10. The signaling of the cytokine IL-4 has previously been shown to have regulatory functions in atherogenesis and has in the same study been suggested to have therapeutic potential in CVD [130]. In contrary, IL- 10 has in several studies been demonstrated to have protective functions in atherosclerosis [85, 137, 188, 224]. However, conflicting results claim that IL-10 does not alter atherosclerosis in mice [207], declaring that regulation and modulation of this disease is highly complex.

After 70 days on a high fat-diet, the amount of plasma cells were significantly higher in the experimental group, as well as the levels of antibodies compared to the control group, indicating that hypercholesterolemia induced a systemic inflammatory response. To investigate the role of the spleen in atherosclerosis, we performed splenectomy on our experimental mice and compared them with sham-operated mice. Interestingly, we saw a decrease of the B1a cells in the peritoneal cavity of splenectomized mice, as well as a decrease in IgM, since B1a cells are the main subset for producing natural antibodies.

In contrast to B2 cells, which are considered to be atherogenic [4], B1a cells have in former studies been shown to have atheroprotective functions [33], due to the secretion of natural IgM antibodies which clear modified low-density lipoprotein, as well as necrotic and apoptotic debris [124]. In the same study, B1a cells were suggested to act as regulatory B cells by the secretion of the anti-inflammatory cytokine IL-10, inhibiting pro-inflammatory cytokines, produced by T cells and macrophages in atherosclerotic lesions. Additionally, others have demonstrated B1b cells to produce atheroprotective IgM antibodies against oxidation-specific epitopes in mice, and proposed a similar mechanism in humans [202].

To further interrogate our findings regarding germinal center formation, and clarify a possible function of germinal centers in atherosclerosis, we performed numerous bone marrow transplants from mice deficient in developing either mature B cells or GC B cells, into irradiated mice of our inducible *Apoe* knockout model. We showed that the loss of mature B cells led to a significant decrease of atherosclerotic lesions in the aorta of experimental mice. Interestingly, we saw a large decrease of atherosclerotic plaques in mice that lacked GCs, proposing the GC reaction to be responsible for pro-atherosclerotic autoimmune responses. Former studies have demonstrated atheroprotective roles for B cell-responses in the spleen [78], yet other have proposed GC formation as pro-atherogenic [40]. The involvement and function of B cells in atherosclerosis and CVD is likely to be B cell subset-dependent [59, 184]. Our findings, supported by previously published data from others, [33] suggest an opportunity to inhibit or reduce atherosclerosis by targeting and dampening the GC-response. This knowledge aid development of future treatments for atherosclerosis and cardiovascular disease.

4 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

The studies in this thesis have aimed at identifying and understanding the involvement of the adaptive immune system and B cells in various autoimmune disorders, by using different genetically modified mouse models. We created and characterized several new murine models, which could be used for future studies as well. We also investigated inducible disease models like EAE and CIA in order to investigate disease course and development. The knowledge gained and the data provided from our studies can be applicable both in future research project, and possibly when developing or refining therapies for autoimmune disorders. The conditional allele of *Arid3a* that we created to interrogate its function regarding B1 and B2 cell fate, has improved our understanding of the transcription factor ARID3A in early and late B lymphopoiesis.

This model did not lack B1 cells, however it had reduced B1a cell numbers in the peritoneal cavity, reduced B1 cells in the bone marrow and expanded numbers of cells in most B2 cell subsets in both bone marrow and spleen, as well as reduced levels of several antibodies in blood plasma, especially those against phosphorylcholine. These findings could be useful in investigating and understanding disease models where natural antibodies are involved, such as SLE [244]. In a future project, it would be of value to study B cell progenitors in the fetal liver of this conditional knockout, since *Arid3a* is highly expressed in this organ according to the ImmGen database [98]. There is a possibility that ARID3A and ARID3B have redundant functions in B lymphopoiesis, hence it could be of interest in setting up a combined deletion of these transcription factors in order to clarify this.

In the second and third study, we applied well-established and accepted disease models of rheumatoid arthritis and multiple sclerosis on two different germinal center deficient strains, and found that the GC reaction can both prevent and promote disease. When inducing CIA, an animal model of RA, in GC deficient mice, we demonstrated that these mice were entirely protected against disease, although they were completely susceptible to CIA. Investigating the disease course of EAE, a rodent model used for studying MS, we found that germinal center B cells were either disease promoting or protecting, in an antigen-dependent manner. In both of these studies, the importance of GCs were elucidated, highlighting the significance of interrogating this B cell subset in future projects.

For forthcoming studies, it could be valuable to inject B cells or antibodies like IgG, in GC deficient mice induced with CIA or EAE, in order to explore and evaluate the disease outcome. It would be interesting to see if the protective function of GC B cells in CIA is abolished after the injection of either B cells or antibodies, and if the effects we saw in the EAE model could be altered. Regarding the GC reaction in general, it could be of interested to study this B cell subset in humans, and possibly target it for more refined therapies, compared to the treatments that currently exist.

The inducible model of hypercholesterolemia and atherosclerosis provide us with a new disease model that can be used for investigating the involvement of the immune system and the development of atherosclerotic lesions in the adult mouse. Our characterization of this mouse model confirmed that GCs and antibodies play significant roles in several disorders, and that targeting this subset in a clinical setting could be of great interest. We have used several advanced methods for characterizing our animal models, as well as for evaluating the effects of disease induction.

It can be challenging to perform research on murine models resembling disease in humans, due to several genetic and physiological differences [183], however animal models are a valuable resource for the studies of autoimmune disease and are more similar to human disorders than can be expected [183]. The findings described in this thesis do not only demonstrate how murine models can be utilized to learn more about immunology and inflammation under various settings, but also contribute significantly to the field of adaptive immunity and autoimmune disease.

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